

Expression of the Gene for Main Intrinsic Polypeptide (MIP): Separate Spatial Distributions of MIP and β -Crystallin Gene Transcripts in Rat Lens Development

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Abstract. The main intrinsic polypeptide (MIP) is the major protein present in the lens fiber cell membrane and is the product of a gene which, as far as is known, is expressed only in the lens. We have used in situ hybridization and immunofluorescence microscopy to characterize the expression of this gene during the course of development in the rat. At progressive stages of lens morphogenesis, we find that synthesis of the protein is closely tied to the accumulation of MIP mRNA in cells that are committed to terminal differentiation, first in the elongating presumptive primary lens fibers and later in the secondary fibers as they differentiate from the anterior epithelial cells. The

transcripts accumulate in the basal cytoplasm of the primary fibers and in the cytoplasm which surrounds the cell nucleus in the secondary fibers. We have compared this pattern of expression with that of a gene for a cytoplasmic protein, β -crystallin β -A1/A3. In sharp contrast to the localized concentrations seen for the MIP mRNA, β -A1/A3 transcripts are relatively uniformly distributed throughout the cytoplasm. Neither MIP nor crystallin gene appears to be transcriptionally active in the undifferentiated epithelial cell, but transcripts from the β -A1/A3 gene appear earlier in fiber cell differentiation than do those from the gene for MIP.

THE developing eye lens is unusually well suited for the study of the mechanisms of gene expression during morphogenesis. The cells of the lens epithelium follow a single pathway of differentiation, from the earliest stages of lens development and throughout life, maturing to become fiber cells which form the mass of the lens. This differentiation of epithelium to fiber, both during embryonic development and in the mature lens, is characterized by the appearance and subsequent massive accumulation of the cytoplasmic crystallins (α , β , and γ in mammals [Harding and Crabbe, 1984]) and of an integral membrane protein known as the main intrinsic polypeptide (MIP)¹ (Alcala et al., 1975; Broekhuysen et al., 1976; Waggoner and Maisel, 1978; Bloemendal, 1979; Vallon et al., 1985). The specific function of MIP in the lens fiber cell membrane is still an unsolved problem. In our previous work (Gorin et al., 1984) we have discussed the controversy surrounding the proposed role of MIP in the formation of the membrane channels for intercellular communication (i.e., gap junctions). The evidence we presented based on cDNA cloning and analysis of the deduced amino acid sequence showed that MIP has the structural characteristics that would be expected if it were a channel-forming protein. Reconstitution experiments support this view (Nik-

aido and Rosenberg, 1984; Gooden et al., 1985; Peracchia and Girsch, 1985; Zampighi et al., 1985).

The gene for MIP does not seem to be expressed in tissues other than the lens, nor has MIP been detected in the membranes of the epithelial cells of the developing or mature lens (Vermorken et al., 1977; Waggoner and Maisel, 1978; Paul and Goodenough, 1983a; Fitzgerald et al., 1983). The specificity of expression of this protein and its presence in homologous if not identical form in lenses from a wide variety of species (Takemoto et al., 1981) suggest that it is of fundamental importance for the normal development and highly specialized function of the lens. By the use of an antisense RNA probe and a polyclonal antibody specific for MIP, we have now been able to follow the pathway of expression of the gene for MIP from DNA to RNA to protein at successive stages of embryonic development. We have compared the spatial and temporal pattern of distribution of the mRNA for MIP with that for a cytoplasmic marker for lens cell differentiation, the β -crystallin polypeptide, β -A1/A3 (Gorin and Horwitz, 1984; McAvoy, 1978a, b; Piatigorsky, 1981) to get a fuller understanding of the sequence of gene activations that leads to lens cell terminal differentiation.

Materials and Methods

Before starting our experiments we carried out a number of preliminary studies to determine conditions that would allow us to achieve simultaneously good preservation of morphology, retention of RNA and protein anti-

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1. *Abbreviations used in this paper:* MIP, main intrinsic polypeptide; RER, rough endoplasmic reticulum.

genicity, RNA probe accessibility, and specificity of binding of RNA and antibody probes. We will discuss these as we describe the methods that we ultimately used to arrive at the results we present here.

Preparation of Tissues

Embryos were dissected from the uteri of pregnant rats (Sprague Dawley, Simonsen Laboratories, Gilroy, CA) after 11–19 d of gestation. Eyes were also dissected from newborn rats 1 and 5 d after birth. The embryos (days 11–15) or, at later stages, dissected eyes, were fixed in Bouin's solution several hours to overnight, washed and dehydrated in several changes of 50% ethanol (ETOH) to remove the picric acid, and then stored in 70% ETOH at 4°C for one to several days. Before embedment in paraffin (Paraplast +), the tissues were dehydrated in graded ethanols, then cleared in two changes of xylene and infiltrated with xylene/paraffin mixed 1:1. Sections 5–10 μm thick, generally cut in a plane parallel to the optic axis, were mounted on glass slides coated with polylysine. The embryos were staged according to the criteria of McAvoy (1978b). Other protocols tried included use of 1% glutaraldehyde in 0.1 M cacodylate buffer, Zenker's solution, or Petrunkevitch's fluid (Jeffery et al., 1983). We also used unfixed eyes frozen in Freon at its melting temperature, then placed in liquid N_2 . Such eyes were sectioned while frozen and then fixed in absolute alcohol. Eye lenses are hard and brittle, and only the treatments with Bouin's or Petrunkevitch's fluid resulted in tissues that could be adequately sectioned and showed no adverse effects on morphology. Overall, Bouin's fixed tissues were judged to be superior, but samples treated with Petrunkevitch's fluid were also processed for *in situ* hybridization with antisense probes for MIP, as shall be described. Patterns of distribution of the ^{35}S -labeled probe after autoradiography were the same, independent of fixative used. Antigenicity of MIP was preserved in the Bouin's fixed tissues.

Preparation of Single-stranded RNA Probes

The 5' Pst I–Hind III fragment of the cDNA clone for MIP described by us earlier (Gorin et al., 1984) was excised from the original pBR322 vector and inserted into the SP 6 transcription vector (Promega-Biotec, Madison, WI) in the proper orientation to transcribe antisense RNA (clone 85). The MIP template was linearized by digestion with Bgl II yielding a transcript of ~400 bases of the 3' end of the coding region. In pilot experiments we also used two other antisense constructs: clone 85 truncated with Xba I to produce a transcript of 900 bases representing the entire coding region and clone 140 (Pst I to Pst I fragment) linearized with Xba I to give a 275-base transcript representing 5' coding sequences. Results of hybridizations were the same regardless of which of the three probes was used. The 800-base-long cDNA clone for bovine β -25/23 crystallin (Gorin and Horwitz, 1984), which is the equivalent of rat β -A1/A3 (Piatigorsky, J., personal communication), is also in the SP 6 vector and was the generous gift of M. Gorin. The β -crystallin template was linearized with Bam HI yielding a transcript which represents the entire coding region. Transcriptions were done under the conditions recommended by Promega-Biotec, in the presence of ^{35}S -UTP (total concentration of UTP, 15 μM). Probes were labeled to sp act $1\text{--}2 \times 10^8$ dpm/ μg . After synthesis and DNase digestion, the RNA probes were adjusted to an average length of 150 bases by alkaline hydrolysis (Cox et al., 1984). The cDNA for MIP was also inserted into the SP 6 vector in the orientation for transcription of mRNA (clone 181) and synthesized as described to be used as a negative control.

In Situ Hybridization

Pretreatment of paraffin sections and hybridization of the sections with the ^{35}S -labeled probes were done with minor modifications of the procedure described by Cox et al. (1984). Briefly, the sections were deparaffinized in xylene, hydrated by passage through graded ethanols, incubated with proteinase K (1 $\mu\text{g}/\text{ml}$) for 30 min at 37°C, then treated with 0.1 M triethanolamine followed by 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature, washed, and dehydrated. The ^{35}S -labeled probes (estimated concentration, 2 $\mu\text{g}/\text{ml}$) were added to a hybridization solution containing 50% formamide, 0.3 M NaCl, 20 mM Tris HCl, pH 8, 5 mM EDTA, 500 μg tRNA/ml, 10% dextran sulfate, 10 mM dithiothreitol, and 0.02% each of BSA, Ficoll, and polyvinylpyrrolidone. For transcription from the templates cloned by G/C tailing, 500 μg poly C/ml was included in the hybridization solution. After the sections were covered with hybridization solution, appropriately cut Saran Wrap was applied to keep the hybridization mix in contact with all sections. The slides were then placed in a humidified chamber for incubation at 50–55°C. Slides were washed and

treated with ribonuclease A (20 $\mu\text{g}/\text{ml}$) also as described by Cox et al. (1984), with final washes in $0.1\times$ SSC at the hybridization temperature. After dehydration, the slides were dipped in NTB 2 (Eastman Kodak Co., Rochester, NY), exposed for 6 d (or for one series of slides, 3 wk), developed in Kodak D-19, then stained with hematoxylin and eosin.

Specificity of Hybridization. Although the results are largely based on hybridization with the 400-base transcript from clone 85 just described, we also tested the 900-base transcript from this clone and a 275-base transcript from clone 140 prepared as just described. Results of hybridization at 50–55°C in 50% formamide as recommended by Cox et al. and others were the same with all the probes. As the hybridization and wash temperatures were raised above 55°C there was a gradual decrease in the signal but no change in the pattern of autoradiographic silver grain distribution. MIP cDNA inserted into the SP6 vector in the orientation for transcription for mRNA (clone 181) was truncated with Eco RI to yield a sense transcript of the entire coding region. No regions of the lens or nonlens tissues were specifically labeled with this probe. Overall labeling was comparable with that observed over non-lens tissues in the sections hybridized under the same conditions with the antisense probes.

Proteinase K Treatment. Treatment before hybridization at a concentration of 1 $\mu\text{g}/\text{ml}$, 30 min at 37°C resulted in completely reproducible patterns of hybridization, independent of the effective level of proteinase K treatment (results were the same on thick or thin sections). Prolonged exposure of the sections to the enzyme resulted in loss of morphological detail in the thinner sections but even then the distribution of the probes was not altered. When sections were treated with proteinase K after hybridization, rather than before, as has been recommended by some, the binding of the probe was highly variable from section to section on the same slide, sometimes but not always binding to such non-lens tissues as portions of the retina. Thus the proteinase K treatment used was sufficient to insure proper probe binding while preserving structure.

Estimation of mRNA Distribution

To estimate the density of silver grains, the same sections were photographed under bright-field and dark-field illumination and printed at a magnification of $500\times$. The bright-field views were used to locate structural landmarks; dark-field views were used for grain count. The areas sampled included non-tissue areas (emulsion) and non-lens tissues. In the lens we counted anterior epithelium and equatorial, anterior, posterior, and central regions of the lens fibers. The number of silver grains/ μm^2 was determined by the use of a program written by Johnson Chung for the Tektronix 4052 computer equipped with a digitizing tablet. Comparison of the binding seen with our antisense probes with that seen for our sense strand control showed no evidence that either gene is expressed in non-lens tissues. There was always some variability in the absolute levels of background labeling when different sections were compared. One reason for some differences between sections was variation in their thickness. Absolute counts also varied because of minor variations in the hybridization signals from one series of hybridizations to another with the same probe. We therefore felt that the most relevant comparison to detect any low level expression (e.g., by the cells of the anterior epithelium) would be grains/ μm^2 associated with non-lens tissues versus grains/ μm^2 over specific areas of interest in the lens, both determined in the same section. The numerical data is not reported except in the case of the anterior epithelium because the data only confirmed microscopic visualization of labeling intensity.

Immunofluorescence

The polyclonal antibody to bovine MIP was generously supplied by J. Horwitz and D. Bok. The antigen had been purified by PAGE, elution of the protein from the gel band, and hydroxylapatite column chromatography as described in Bok et al. (1982). Before use for immunostaining of tissues, the antibody was tested on Western and dot blots for reactivity to MIP and lack of activity against the water soluble lens proteins which consist predominantly of the crystallins. Sections adjacent to those used for *in situ* hybridization were mounted on glass slides, deparaffinized, hydrated, and immersed in blocking solution (0.2% BSA, 0.1% Triton X-100, 1% normal goat serum in PBS). After testing at dilutions of 1:10, 1:50, 1:500, and 1:1,000, the sections were incubated with the anti-MIP antibody diluted 1:500 in blocking solution, washed, and incubated with fluorescein-conjugated goat anti-rabbit IgG (Gibco, Grand Island, NY) also at a dilution of 1:500. No binding was seen with preimmune serum when it was used at 1:50 dilution nor with second antibody when it was used in the absence of the primary

Results

Spatial and Temporal Patterns of Distribution of MIP and Its mRNA

Lens formation (diagrammed in Fig. 1) begins in the rat at 11 d of embryonic development as the ectodermal cells that overlie the optic vesicle increase in height. The future lens cells then invaginate and pinch off from the surface ectoderm to form the lens vesicle. As lens morphogenesis proceeds, MIP is first detectable by immunocytochemistry as a faint fluorescence along portions of the membranes of some cells of the posterior wall of the early lens vesicle at about day 12.5 of development (data not shown). This corresponds to a time when the cells are elongating rapidly as they differentiate into the primary lens fiber cells. At this stage, only a few cells show a reaction for MIP. In adjacent sections hybridized with the probe for MIP mRNA, no accumulation of autoradiographic grains is detectable above background. By day 13 when the lens cavity has nearly been obliterated (Fig. 1), the membranes of many of the primary lens fibers have become reactive to the antibody (Fig. 2 A). The pattern of stain is not uniform. Some cell membranes are strongly fluorescent, some less so; others are negative. In the positive cells, the protein appears to be most concentrated at the base of the fibers, the regions closest to the developing neural retina. In adjacent sections hybridized with the antisense probe for MIP mRNA (Figs. 2 B and 3 A), the silver grains reflecting the distribution of the mRNA are found to be most dense along the posterior border of the lens vesicle, decreasing in density from the base to the apex of the fibers. The density of the silver grains over the epithelial cells that form the anterior wall of the lens is no different from background (Table I). Thus both the message and the protein are predominantly localized in the basal portions of the primary lens fiber cells.

By day 14 of development, the primary lens fibers have elongated sufficiently to make contact with the anterior epithelium. Cell divisions and differentiation of the anterior epithelial cells that lie near the equator of the lens (Fig. 1) are now said to produce "secondary" fibers. MIP, instead of having the patchy distribution it showed earlier, is found throughout the plasma membrane of the primary fibers and has begun to appear in the membranes of the secondary fibers as they form in the zone of elongation (Fig. 2 C). As was the case during differentiation of the primary fibers, the protein is detectable in the membranes of the secondary fibers only well after the process of cell elongation has begun. The labeled transcripts of the MIP gene are still heavily concentrated at the posterior pole of the lens but are now also seen in the differentiating secondary fibers (Fig. 2 D) where the silver grains are predominantly distributed over the region of the cell containing its nucleus and surrounding cytoplasm (refer to Fig. 1 for location of cell nuclei).

As the lens increases in size and the fibers formed during earlier stages of lens morphogenesis become compacted in the center of the lens, the highest density of grains is over the nuclear and juxtannuclear regions of the elongating secondary fibers. This is evident in sections viewed by bright field (Fig. 2 E), but the intensity of the signal in these regions

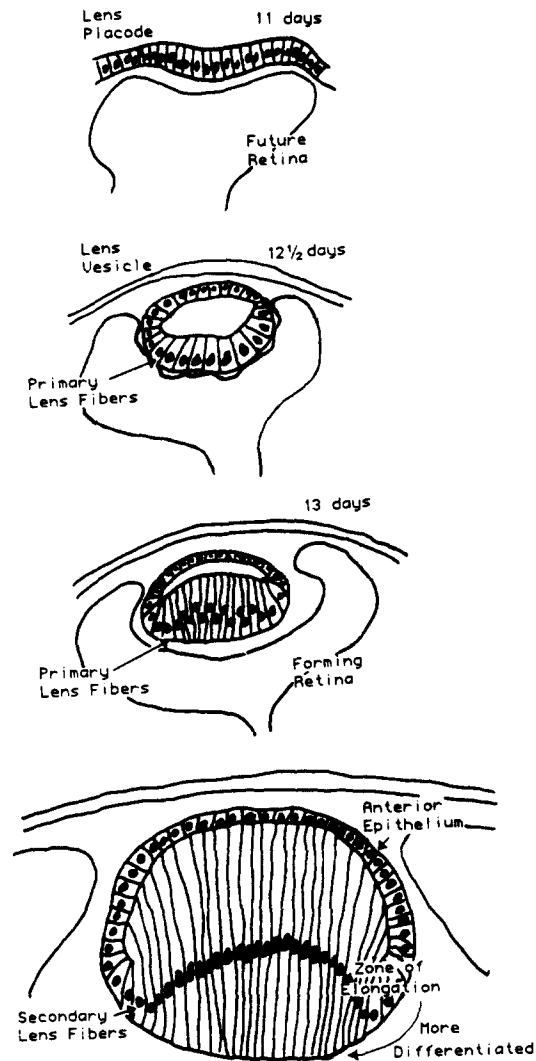
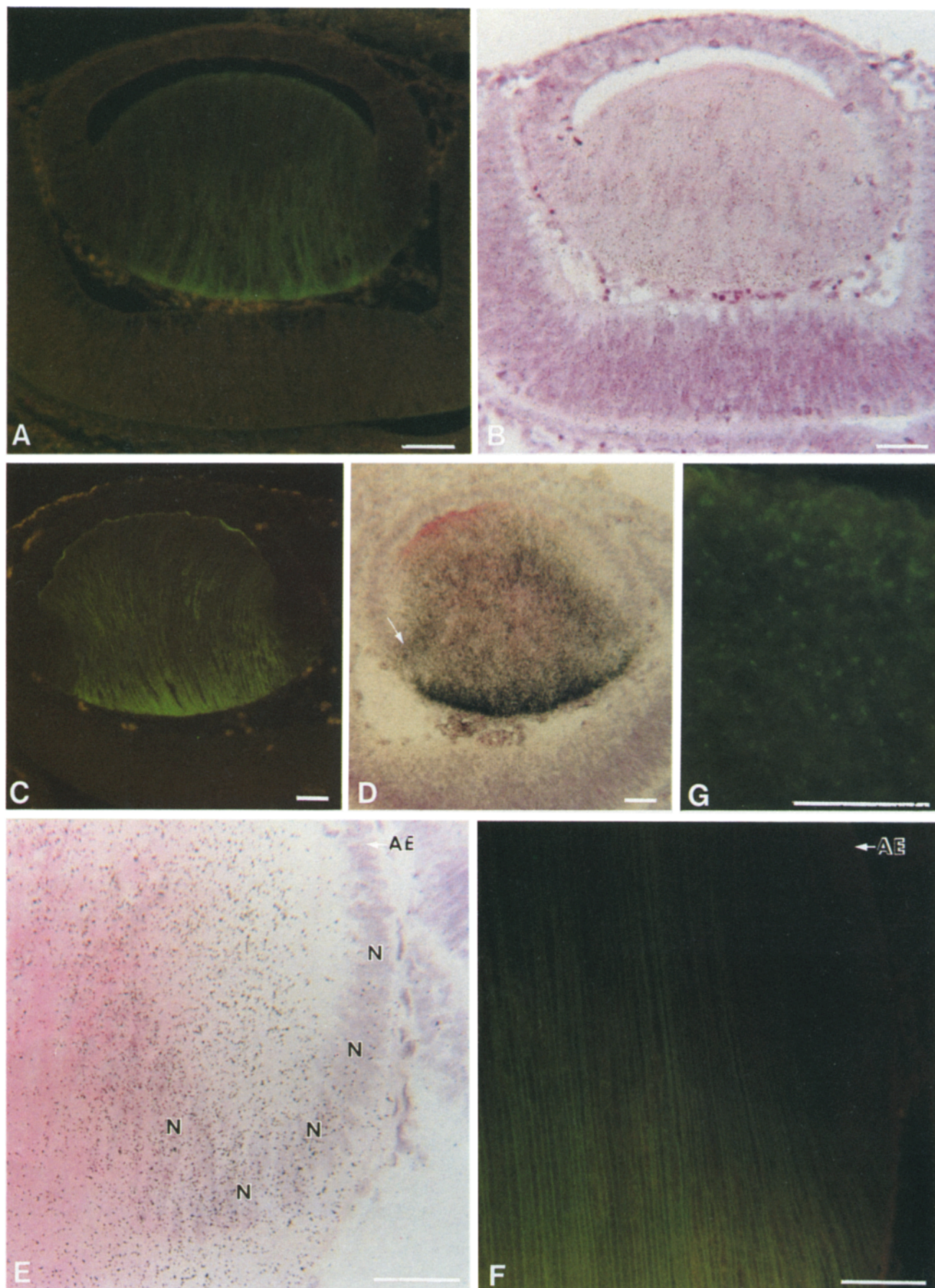


Figure 1. Diagram of the early stages of lens morphogenesis in the rat. The lens placode that forms as a thickening of the ectoderm overlying the optic vesicle invaginates to form the lens vesicle. The cells of the posterior wall of the vesicle differentiate to form the elongated primary lens fibers. These eventually contact the cells of the anterior wall which remain as a simple cuboidal epithelium. Further growth of the lens occurs through division and subsequent differentiation of those anterior epithelial cells which lie near the equator. By this process, successive concentric layers of secondary fibers come to surround the earlier differentiated primary fibers. Heavy black dots represent the nuclei of the cells.

can best be appreciated in dark field (Fig. 3, C and E). The autoradiographic signal decreases over the fibers that lie progressively deeper in the lens so that grain densities over the central region of the fiber mass are only slightly above background. At these later stages as in earlier ones, the density of grains over the anterior epithelium is not significantly above background (Table I). Soon after the MIP message begins to accumulate in the differentiating secondary fibers in the zone of elongation, specific fluorescence is found in the plasma membrane, first at the base of the cell, then spreading throughout the membranes as the fibers are displaced toward the center of the lens (Fig. 2 F). Punctate areas of intense staining now become evident. These areas are seen in lon-



itudinal sections but are most clearly demonstrated in cross sections of the lens cortex (Fig. 2 G). This pattern of distribution of MIP and its message persists in the lens at least until 5 d after birth.

At embryonic stages of development days 11 through 15, entire embryos were sectioned and samples reacted with the antibody and RNA probes. There was no immunofluorescent staining of tissues other than the lens and no noticeable accumulation of silver grains over non-lens tissues.

Differential Expression of the MIP and β -Crystallin Genes

To determine whether the patterns of localization of mRNA just described are specific for the MIP probe, sections from the same embryos were hybridized with the probe for one of the β -crystallins, β -A1/A3. We found distinct differences in the distributions of the two probes but also some similarities. At \sim 13 d of embryonic development, when the message for MIP is first detected as a heavy deposition of silver grains along the posterior border of the lens, the transcripts of the β -crystallin gene are distributed more or less uniformly throughout the cytoplasm of the differentiating primary lens fibers (Fig. 3, A and B). As the secondary fibers form in the zone of elongation in more mature lenses, the autoradiographic grains reflecting β -A1/A3 transcripts remain relatively homogeneously distributed throughout the cytoplasm (Fig. 3, D and F). Compared with the distribution seen for the MIP probe at these later stages, relatively fewer grains are deposited over the cell nucleus (compare Fig. 3, E and F). As is the case for MIP, no β -crystallin transcripts can be detected in the cells that form the anterior wall of the lens vesicle (Table I) and less probe binds to the oldest fibers, near the center of the lens.

During the entire period examined, there are no local concentrations of β -crystallin mRNA, a situation very different from the pattern seen for MIP transcripts (compare Fig. 3, A and B, C and D, E and F). Another major difference in the distribution of the two types of transcripts can be seen in the zone of elongation where the secondary fibers are differentiating (Fig. 4, A and B). The gene transcripts for β -crystallin appear in cells at an earlier stage of differentiation than do the transcripts for MIP.

Table I. Density of Silver Grains over the Lens Anterior Epithelium Compared with That over Non-lens Tissues

Probe	Days of development	Silver grains $\times 10^{-2}/\mu\text{m}^2$, mean \pm SD	
		Anterior epithelium	Nonlens tissues
MIP	12	1.2 \pm 0.3	1.0 \pm 0.4
	12	1.1 \pm 0.5	0.9 \pm 0.1
	12	1.2 \pm 0.4	1.1 \pm 0.3
	12	0.8 \pm 0.3	0.9 \pm 0.1
	22	0.7 \pm 0.4	0.6 \pm 0.2
β -A1/A3	12	1.0 \pm 0.1	1.5 \pm 0.4
	12	0.6 \pm 0.1	0.7 \pm 0.5
	12	0.7 \pm 0.2	1.0 \pm 0.3
	18	0.8 \pm 0.1	0.6 \pm 0.2
	18	0.8 \pm 0.2	1.0 \pm 0.6

Samples from days 15, 17, 19 show similar grain densities.

Discussion

Within the limitations of the commonly used methods we employed in our studies, the main conclusions we can draw are (a) the genes for MIP and β -A1/A3 are not transcriptionally active in undifferentiated lens epithelial cells (or the transcripts are rapidly degraded and not detectable as a result); (b) activation of the β -A1/A3 and MIP genes occurs sequentially in the elongating fiber cells which have already started to differentiate; (c) the expression of the gene for MIP is probably regulated at the level of transcription in the developing lens; (d) there is a mechanism that directs the mRNAs for the two genes to different regions of the cytoplasm of the differentiating fibers; (e) there is progressively less MIP or crystallin message in the older fibers. The hybridization signal seen for MIP is unlikely to represent any but MIP gene transcripts for several reasons. (a) The conditions for hybridization and washes are at the upper limits of stringency commonly recommended for detection of specific RNA-RNA hybrids; (b) the results are indistinguishable with antisense transcripts from three different regions of the MIP cDNA template (see Materials and Methods); (c) Southern blot analysis indicates that MIP is encoded by a single-copy gene (Gorin et al., 1984); and (d) there is no evi-

Figure 2. Spatial and temporal patterns of distribution of MIP and its mRNA. (A, C, F, and G) Sections reacted with anti-MIP and fluoresceine isothiocyanate-conjugated goat anti-rabbit IgG. (B, D, and E) Sections adjacent to A, C, and F but hybridized with the ^{35}S -labeled antisense RNA probe. B and E were exposed for autoradiography for 6 d, D for 3 wk. All photographs are oriented so that the anterior epithelium of the lens is toward the top of the page. (A and B) Sections from day 13 embryo; late lens vesicle stage. The cells of the posterior wall of the lens vesicle have elongated as they differentiate to form the primary lens fibers. The cells of the anterior wall have maintained their cuboidal profile. MIP is detected only in the plasma membrane of the presumptive primary lens fibers (A). Immunofluorescent labeling is most intense along the membrane which surrounds the basal cytoplasm. In B, the concentration of silver grains along the posterior region of the lens indicates that the MIP-coding mRNA is also predominantly localized in the basal cytoplasm of the differentiating fibers. (C and D) 14-d lens. The immunofluorescence (C) indicates that MIP is distributed throughout the plasma membrane of the primary fibers which form the mass of the lens and is accumulating in the membranes of the secondary fibers which are beginning to differentiate from the cells of the anterior epithelium at the equator of the lens. The mRNA transcripts (D) are still predominantly localized in the basal cytoplasm of the primary fibers but are also detected in juxtanuclear regions of the differentiating secondary fibers (arrow). (E and F) 19-d lens, zone of elongation. AE, anterior epithelium; N, nuclei of elongating fiber cells. As the secondary fibers differentiate, the density of the silver grains indicates that the mRNA is most concentrated in the cytoplasm which surrounds the cell nucleus (stained a faint purple), accumulating at or near the time MIP first appears in the plasma membrane near the posterior end of the lens (F). (G) Immunofluorescent staining of cross-sectioned fibers in the cortex of the 18-d lens. The entire plasma membrane is fluorescent but plaque-like areas are more intensely stained. Bars, 100 μm .

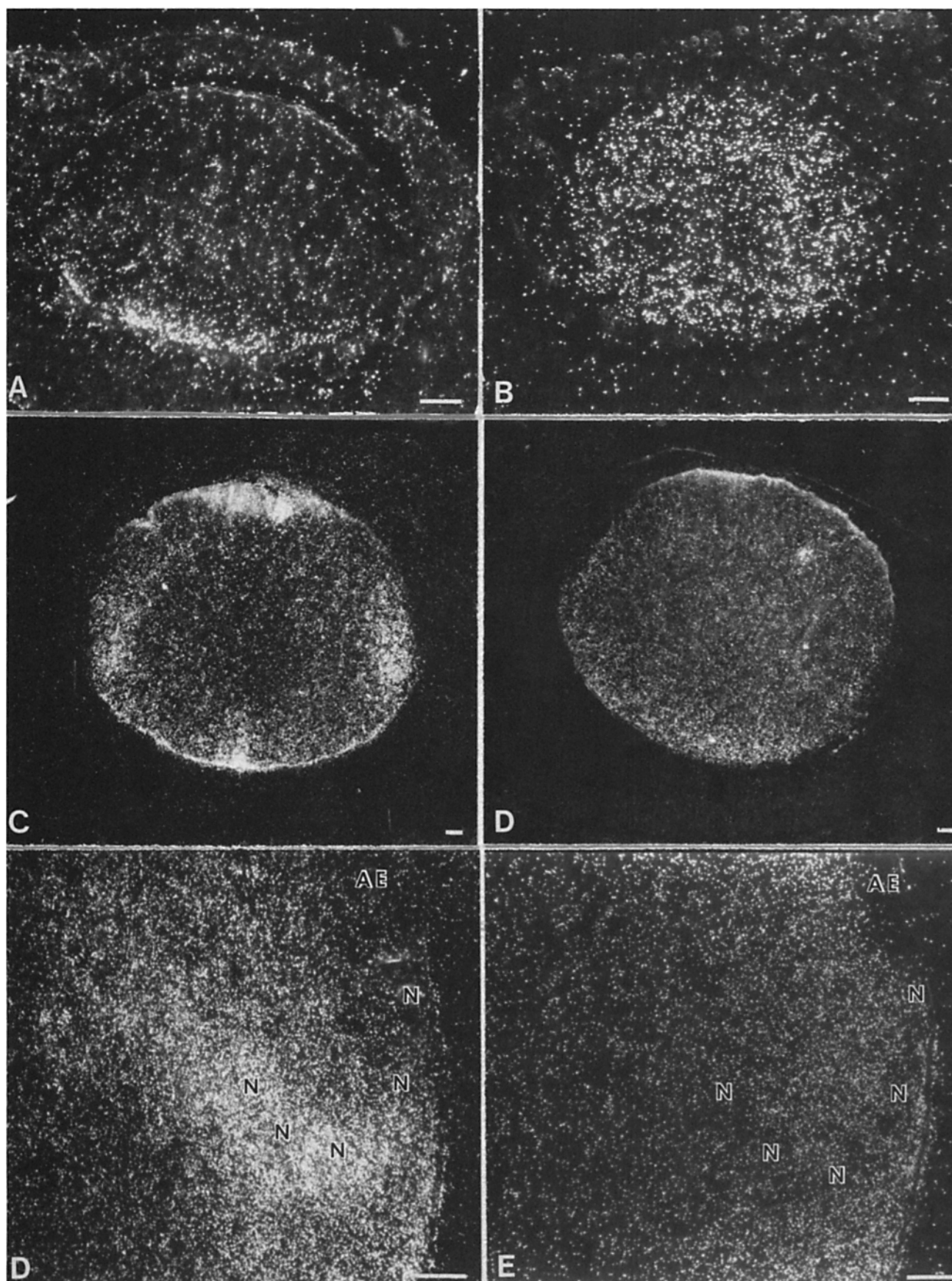


Figure 3. Comparison of the distributions of MIP and β -A1/A3 transcripts during lens morphogenesis. Sections were photographed in dark-field illumination; the anterior epithelium is toward the top of the page. (A, C, and E) Sections from 13- and 15-d embryos and from newborn animals hybridized with the probe for MIP. (B, D and F) Sections from the same stages hybridized with the probe for β -A1/A3. Exposure

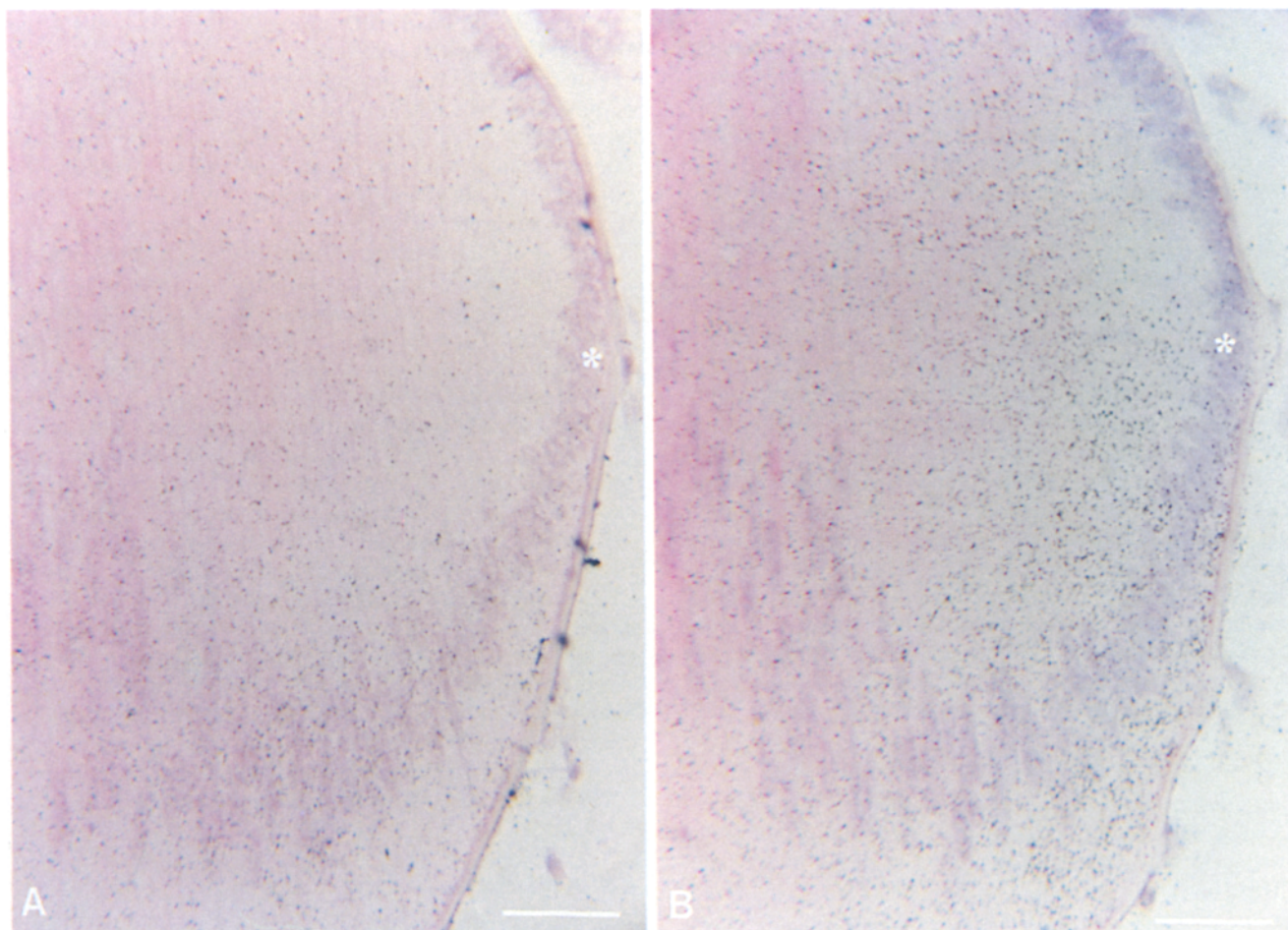


Figure 4. Initiation of MIP and β -A1/A3 gene expression during differentiation of epithelial cells to fiber cells in the zone of elongation. (A and B) Sections from the same lens of a neonatal rat exposed for autoradiography 6 d. (A) Hybridization with the probe for MIP. (B) Hybridization with the probe for β -A1/A3. Transcripts of the β -A1/A3 gene (B) accumulate in cells that are just differentiating from the anterior epithelium (*), whereas the same cells (*) hybridized with the probe for MIP (A) have only background labeling. Cells that have differentiated further show the presence of both types of transcripts. Bars, 100 μ m.

dence of any homologous proteins either at the nucleotide or amino acid sequence level. There are, however, proteins present in the lens fiber cells which are homologous to β -A1/A3. The different β -crystallin polypeptides show only ~40–60% homology (Berbers et al., 1984; Piatigorsky, 1984) and the stringency of our hybridizations should allow detectable binding only to the message for β -A1/A3.

Transcription and Translation of MIP in the Developing Lens

Superposition of the patterns of distribution of MIP and its mRNA at identical stages of development of the eye lens suggests that transcription and translation are intimately cou-

pled as synthesis of MIP is initiated in the differentiating fiber cells. Transcription of the gene for MIP and synthesis of the protein are first detected only after the cells of the posterior wall of the lens vesicle have already begun the dramatic changes that will lead to formation of the primary lens fibers (McAvoy, 1978b). Because of the negligibly low background staining we achieved with the antibody probe, we were actually able to detect the synthesis of the protein in a few of the presumptive primary lens fibers by day 12.5 of development, before any intracellular accumulation of mRNA could be seen by our techniques. At this stage, cells in the posterior wall of the lens vesicle are differentiating asynchronously (McAvoy, 1978b); activation of the gene for MIP is therefore unlikely to occur simultaneously in all cells of

for autoradiography was in each case 6 d. Transcripts for MIP as well as those for β -A1/A3 are detected in the elongating primary (A and B) and secondary fibers (C–F). The transcripts for β -A1/A3 are relatively uniformly distributed in the cytoplasm (B, D, and F). Neither transcript is found in the anterior epithelium which shows only background labeling. Transcripts for MIP are localized in the basal cytoplasm of the primary fibers (A) and in the juxtannuclear cytoplasm of the secondary fibers (C and E). AE, anterior epithelium; N, location of nuclei of the elongating fibers identified in bright field views of the same lens. The bright area at the anterior portion of the lens fibers in C and the bright spot and thin bright line at the anterior portion of the lens fibers in D do not represent a high concentration of autoradiographic grains but reflect defects in the sections. Bars, 100 μ m.

the population. A relatively low level of accumulation of mRNA transcripts in a few of the cells probably does not produce a high enough signal to be detected by in situ hybridization (Anderson and Axel, 1985; Weir and Kornberg, 1985).

There appears to be little or no lag time between detectable accumulation of MIP gene transcripts and the translation of the processed message in the differentiating secondary fibers in the zone of elongation in more mature lenses. An increase in the number of mRNA transcripts followed directly by increased synthesis of the protein is usually taken as an indication of regulation at the level of transcription. We have no way of knowing from the present observations what fraction of the message we detect is in translatable form or how much may be in storage as nontranslating mRNP (Arnstein, 1982). We must also concede the possibility of some level of post-transcriptional control either through an increase in mRNA stability which may occur for some of the mRNAs in fibers at early steps of differentiation of the lens (see Piatigorsky, 1981, and Harding and Crabbe, 1984 for reviews) or at the stage of mRNA processing. In fact, in the secondary fibers a greater proportion of the transcripts for MIP are localized over the cell nucleus than is the case for β -A1/A3 transcripts, suggesting that there may be some element of control of gene expression at the level of mRNA processing and transport from the nucleus (Woodland and Old, 1984).

Expression of the β -A1/A3 and MIP Genes

The genes for β -A1/A3 and MIP appear to be sequentially induced at about the same stage of morphogenesis in the lens vesicle. We were not able to pinpoint the exact relationship at these earlier stages, but in slightly older lenses it appears that the β -crystallin gene is already transcriptionally active when expression of the gene for MIP is induced. The β -A1/A3 transcripts accumulate in the presumptive fibers that lie at the periphery of the lens and are just beginning to elongate. MIP transcripts accumulate only after the cells have further elongated and have been displaced from the periphery toward the center of the lens. The decrease in number of transcripts in cells lying progressively deeper in the center of the lens is seen for both MIP and β -A1/A3 and probably reflects the gradual decrease in RNA synthesis that generally occurs with the degradation of the cell nucleus and other organelles during terminal differentiation of the lens fibers (Willis et al., 1969; Modak and Perdue, 1970; Kuwabara, 1975).

The cells of the anterior epithelium appear to be devoid of either MIP or β -A1/A3 transcripts at all time points studied. We, as others (Waggoner and Maisel, 1978; Vermorken et al., 1977; Broekhuysen et al., 1979; Paul and Goodenough, 1983a; Vallon et al., 1985), also found no suggestion that MIP is present in the membranes of the epithelial cells. This lack of immunostaining of epithelial cell membranes is particularly striking at the early stages of lens morphogenesis before the cells of the posterior wall fill the lumen of the lens vesicle and make solid contact with the anterior epithelial cells. MIP is present in fiber cell membranes only well after the process of terminal differentiation has begun (Vermorken et al., 1977; Broekhuysen et al., 1979; Bloemendal, 1979). Because either protein synthesis or mRNA transcription at very low levels would not be seen by our methods, we still cannot state absolutely that MIP is exclusively a product of lens fiber cells, but the timing of the appearance and accumulation of both

the message and the protein strongly suggest that this is the case, at least for the fetal lens.

Our results indicate that the gene for β -A1/A3 is also first expressed in cells that are already committed to differentiate because they lie in the equatorial zone where the cells have undergone their final mitotic division (McAvoy, 1978b). Piatigorsky and his colleagues (Hejtmancik et al., 1985) have recently detected mRNA for some of the β -crystallin polypeptides not only in fiber cells but in cells located in both the central and equatorial epithelium of the developing chick lens. These findings do not seem to be inconsistent with immunochemical and biochemical data on the synthesis of the β -crystallins in chick lens as reviewed by Piatigorsky (1981) or by Harding and Crabbe (1984). It is possible that we have missed some low level or transient expression of β -A1/A3 in the more central epithelial cells of the rat lens. However, the spatial and temporal distribution we observed for the message for β -A1/A3 closely matches the pattern of distribution of β -crystallin polypeptides in the primary and secondary fibers of the developing rat lens as described by McAvoy (1978b). Admittedly, in the latter study the immunofluorescence reflects the distribution of antibodies raised to a mixture of diverse β -crystallin polypeptides and it is not known whether they cross-react with β -A1/A3. Nevertheless, as it now stands, it seems most likely that any seeming discrepancy is a reflection of the species differences that have been described for the patterns of expression of various crystallin polypeptides (McAvoy, 1978a and b; Piatigorsky, 1981; Harding and Crabbe, 1984).

Cellular Localization of Transcripts

The differential distribution of the mRNAs for MIP and β -A1/A3 is striking. The message for β -A1/A3 is distributed throughout the fiber cell cytoplasm at all stages examined in contrast to the highly localized concentrations of the mRNA for MIP, initially in the cytoplasm at the base of the presumptive primary fibers and at later stages in juxtanuclear regions of the cytoplasm of the differentiating secondary fibers. The specificity in the intracellular distributions of these two different species of mRNAs suggests that the patterns reflect the processes of synthesis of these membrane (MIP) and cytoplasmic (β -A1/A3) proteins. Because polyribosomes that would presumably be the sites of synthesis of the β -crystallins are dispersed throughout the cytoplasm of the differentiating fibers (our unpublished observations; Willis et al., 1969) as is the mRNA, interpretation of the distribution, at least of the actively translating β -A1/A3 mRNA, seems relatively straight forward. The situation for MIP is more complex. The highly regionalized intracellular distribution of this message suggests that it must be largely associated with some structure or organelle. A natural candidate for targeting of the message for this membrane protein, which in vitro is cotranslationally inserted into microsomes (Paul and Goodenough, 1983b; Anderson et al., 1983), would be the rough endoplasmic reticulum (RER) (Blobel, 1980). At ~13 d of development in the rat, when we first see accumulation of MIP transcripts at the base of the differentiating primary fibers and the protein is most abundant in the plasma membrane at the base of the cells, there does appear to be a parallel segregation of RER and mitochondria although the number of profiles of RER seen is quite variable from cell to cell

(our unpublished observations). In differentiating secondary fibers the RER appears to be concentrated in the perinuclear cytoplasm (Piatigorsky et al., 1972; Wanko and Gavin, 1959). But the RER is never very abundant and it is possible that some other association is involved, perhaps an association with the cytoskeletal framework (Jeffery, 1984). From their studies on the in vitro synthesis of MIP, Ramaekers et al. (1980) concluded that the mRNA supporting the synthesis of MIP was preferentially associated with the fiber cell plasma membrane-cytoskeleton complex in the bovine lens, but this has not been confirmed by Hentzen et al. (1984). More recently, Dunia et al. (1985) have suggested that the message might be transcribed on vesicles of RER closely associated with the plasma membrane. In view of the limited resolution of autoradiography, we cannot distinguish between these alternatives. We can be certain, however, that the whole cell membrane is not involved. Because the other studies were concerned with the distribution of the message in adult lenses, it is possible that the message is associated with some cytoskeletal framework or the RER that is redistributed with aging of the lens. These questions would best be answered by application of our probes to thin sections for electron microscopy.

Is MIP a Gap Junction Protein?

Although our study was not designed to address the question of whether or not MIP is a component of the gap junction-like structures that interconnect the lens fibers, some of the observations we have made here suggest a comment on this unresolved problem. Gap junction-like structures first appear in the fiber cell plasma membrane at about the same stage of differentiation as MIP synthesis is amplified (Benedetti et al., 1974; Kuzak et al., 1980; Schuetze and Goodenough, 1982; Lo and Harding, 1986) and there is evidence that anti-MIP antibodies block gap junction-like channel activity in reconstituted systems (Gooden et al., 1985). In the study we present here, the pattern of immunofluorescent staining we observe on the fiber cell membranes suggests that the antibody is binding not only throughout the membrane but with greater avidity to plaque-like areas of the membrane, particularly in the lens cortex. Alternately, this punctate staining could reflect regions of higher protein concentration. The distribution of antibody binding sites is thus most compatible with the view that MIP is present in both the nonjunctional and the junctional membrane, as proposed from immunocytochemical studies by electron microscopy of intact lenses (Fitzgerald et al., 1983; Vallon et al., 1985) and of isolated junctions (Bok et al., 1982; Sas et al., 1985). Interestingly, Paul and Goodenough (1983a) also found that by immunofluorescence MIP appeared to be present throughout the fiber cell membrane, junctional and non-junctional, but failed to detect it in junctions in the isolated membranes. A protein of 70 kD and immunologically unrelated to MIP also apparently localizes to lens fiber junctions by both immunofluorescence and electron microscopy (Kistler et al., 1985; Gruijters et al., 1987). This protein is apparently not present in the nonjunctional membrane so that it appears to be somehow specifically involved in junction structure. How or whether MIP or the 70-kD protein interact to form the membrane-to-membrane channels known to interconnect the lens fibers (Kuzak et al., 1985) is still un-

certain. The occurrence of multiple proteins in gap junctions recently reported by us in the case of the liver (Revel et al., 1987; Nicholson et al., 1987) may be relevant in understanding the structure of lens junctions.

We wish to thank Brian Austin for his excellent technical assistance, Rob van Leen for his helpful suggestions, and George Diaz for his expertise with the color prints.

The research was supported by Research Grant GM-34833 from the National Institutes of Health.

Received for publication 1 September 1987, and in revised form 29 October 1987.

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